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Genomic characterization of virulent, attenuated, and revertant passages of a North American porcine reproductive and respiratory syndrome virus strain

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Abstract

Pigs were exposed to three passages of the NADC-8 strain of porcine reproductive and respiratory syndrome virus (PRRSV) to investigate the relationship between genotypic and phenotypic properties. Differences were found in the virulence of the three passages called virulent, intermediate, and avirulent. Avirulent virus was derived by attenuation of virulent virus in cell culture and intermediate virus was derived by passage of avirulent virus in a pig. Nucleotide sequence differences between virulent and avirulent virus consisted of 50 nucleotide changes and a three-nucleotide deletion, and between avirulent and intermediate virus consisted of 8 nucleotide changes resulting in six amino acid changes. Three of these amino acid changes were direct reversions to virulent virus. Genetic changes, especially those seemingly associated with attenuation followed by some degree of reversion to virulence, in ORF1a, ORF1b, and ORF 6 regions of the genome may be involved in the control of PRRSV replication and virulence.

Keywords: NADC-8 strain; Virulence; Passages

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a disease of swine characterized primarily by reproductive failure in pregnant gilts and sows and respiratory tract illness in pigs of all ages, but especially in suckling and nursery-age pigs. The causative agent, PRRS virus (PRRSV), was discovered in 1991 (Wensvoort et al., 1991) and is classified as a member of the *Arteriviridae* virus family in the order *Nidovirales* (Cavanagh, 1997) PRRSV is an enveloped virus with a positive-sense, single-stranded RNA genome approximately 15.1–15.5 kb long (Meulenberg et al., 1993; Shen et al., 2000). The genome is organized in a 5′ to 3′ orientation as follows: a 5′ capped structure, 5′ non-translated leader sequence, open reading frames (ORFs) 1a, 1b, 2–7, 3′ non-translated region, and a polyadenylated tail. ORF1a and 1b are overlapping and comprise about 80% of the genome. They are believed to encode nine and four nonstructural proteins (Nsp), respectively, involved with viral replication and transcription (Allende et al., 1999; Meulenberg et al., 1993; Nelsen et al., 1999). The remaining genome, ORFs 2–7, encodes the PRRSV structural proteins. The structural proteins are translated from a nested set of 3′ coterminal subgenomic mRNAs.

Genetic analysis of PRRSV isolates has revealed two predominant genotypes, the European and North American genotypes. Based on data from the original European and North American prototype PRRSV isolates, there was 55–80% homology at the nucleotide level for various structural genes (Murtaugh et al., 1995). Analysis of additional

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PRRSV isolates indicated the European isolates appeared more homogenous as a genotype than the North American PRRSV isolates (Le Gall et al., 1998; Mardassi et al., 1995; Meng et al., 1995; Suarez et al., 1996). However, recent studies have revealed more and more genetic heterogeneity in European PRRSV isolates that may be attributed to more extensive testing of isolates from a larger geographic region (Forsberg et al., 2002; Indik et al., 2000; Stadejek et al., 2002) and to the introduction of a North American PRRSV isolate (an attenuated PRRSV vaccine) (Madsen et al., 1998).

In 1996, a PRRSV vaccine program was initiated in Denmark for the control and prevention of PRRS (Botner et al., 1997). The vaccine used was a commercially available attenuated-virus vaccine derived from a North American field isolate that was attenuated by repetitive passage in cell culture (Yuan et al., 2001). Following the implementation of the vaccination program, epizootics of PRRS-like disease were reported in some of the vaccinated herds and PRRSV was isolated from clinically affected swine. In some cases, genetic analysis indicated the PRRSV was closely related to the vaccine virus (North American genotype) and not the Danish field viruses (European genotype) (Madsen et al., 1998). Subsequent studies demonstrated the vaccine-like PRRSV isolates were pathogenic (Nielsen et al., 2002). Collectively, this evidence supports the theory that the attenuated PRRSV vaccine virus had mutated following replication in swine and this swine-passaged vaccine virus had gained virulence. Common genetic changes were found among several Danish vaccine-like PRRSV field isolates when the nucleotide sequence of the structural genes were determined (Nielsen et al., 2001; Storgaard et al., 1999). Some of these changes involved a nucleotide change that lead to a "reversion" from the sequence of the attenuated virus vaccine to that of the wild-type parental PRRSV. These changes are presumed to be related to the reported increased virulence of the vaccine-like PRRSV isolates. Additional studies reporting full-length genomic sequences have been completed that strived to relate genetic changes to virulence. One report compared the vaccine nucleotide sequence with its parental strain, VR-2332 (Yuan et al., 2001), and a second report compared the nucleotide sequence of a virulent field isolate, believed to be vaccine related, with the vaccine (Allende et al., 2000). Although genetic differences were found in both studies, there was no apparent common denominator that related genetic change with virulence.

In the study reported here, we investigated further the relationship of genetic change and virulence through a series of in vitro and in vivo experiments. A wild-type PRRSV was rendered avirulent by 251 passages in cell culture and then this virus was passed in a pig in an attempt to force a reversion of the attenuated virus to a more virulent state. The genetic changes associated with attenuation of the PRRSV strain and the partial reversion to virulence of this strain are reported. The relationship of the genetic changes to attenuation and reversion and the similarities of these genetic changes to what has been previously reported are discussed.

Results

Animal inoculations

Based on previously described animal studies (see Materials and methods), there appeared to be in vivo phenotypic differences among the three different passages of the NADC-8 PRRSV strain. The objective of the animal study was to compare the virulence of these three passages under like conditions. Passage level 2 virus [NADC-8 (2)] was believed to be more virulent than the NADC-8 (252P) virus, and the NADC-8 (251) virus was considered the least virulent of the three. No PRRSV, PRRSV-specific antibody, or clinical disease was detected in Group 1, the sham-inoculated controls, and in Group 2, the group inoculated with the (251) virus (Table 1). In contrast, virus and virus-specific antibody were detected in Group 4 pigs, the pigs inoculated with the NADC-8 (2) virus. In addition, moderate clinical disease consisting of mild dyspnea and intermittent anorexia and listlessness was observed in some or all of the pigs from day 3 through about day 10 of the experiment. Results from Group 3, pigs inoculated with the (252P) virus, were intermediate in that virus and virus-specific antibody were detected in some of the pigs and no clinical disease was observed. When compared under similar conditions, the

Table 1
In vivo comparison of NADC-8 PRRSV strain passages (2), (251), and (252P)

Group*	Experiment day			
	3**	10	17	35
(A) Virus isolation				
Sham	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)
(251)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)
(252P)	1/4 (0.4)	1/4 (0.2)	2/4 (0.9)	0/4 (0.0)
(2)	3/4 (2.8)	4/4 (3.6)[a]	3/4 (3.0)	0/4 (0.0)
(B) Serology				
Sham	0/4 (0.000)	0/4 (0.000)	0/4 (0.000)	0/4 (0.000)[c]
(251)	0/4 (0.000)	0/4 (0.000)	0/4 (0.000)	0/4 (0.000)[c]
(252P)	0/4 (0.000)	0/4 (0.000)	1/4 (0.317)	3/4 (0.850)[b,c]
(2)	0/4 (0.000)	3/4 (0.904)[a]	4/4 (0.766)	4/4 (1.280)[a,b]

* Each group of pigs (n = 16) on experiment day 0 was exposed to sham inoculum, passage (251) virus, passage (252P) virus, or passage (2) virus. Four pigs from each group were euthanized on Experiment Days 3, 10, 17, and 35 and their serum tested for PRRSV (A) and PRRSV-specific antibody (B). No PRRSV nor antibody were detected in Day 0 serum samples from any pig (data not shown).

** Experimental day pigs necropsied. (A) Number of pigs positive for virus/number of pigs tested for virus (mean virus titer − $\log_{10}$). (B) Number of pigs positive for PRRSV antibody/number of pigs tested for antibody. Positive determined as ELISA S/P ratio ≥0.4, (mean S/P ratio). Superscript letter denotes statistical difference among groups on a specific day, for example, a is different from b which is different from c.

Table 2
Summary of all nucleotide and amino acid changes among passages 2, 251, and 252P of PRRSV strain NADC-8

Gene	Protein	Nucleotide position	NADC-8 (2)		NADC-8 (251)		NADC-8 (252P)	
			N	AA	N	AA	N	AA
ORF1a	Nsp1	755	G	D	A	N		
		801	G	R	A	H		
		837	A	E	A	E	G	G
		840	G	G	G	G	T	V
	Nsp2	1628	A	I	G	V		
		1877	A	E	G	G		
		1978	C	H	Y(T/C)	H		
		2042	R(G/A)	E/K	G	E		
		2341	G	L	G	L		
		2504	G	D	A	N		
		2607	A	N	C	T		
		2740	G	P	A	P		
		2800	T	D	C	D		
		3166	C	D	Y(T/C)	D		
		3245	G	A	A	T		
		3763–3765	TTA	L	Deletion	Deletion	Deletion	Deletion
		3767	T	F	A	L		
		3914	T	L	C	L		
	Nsp3	4379	T	C	C	C		
		4709	C	V	T	V		
		4777	G	R	A	K		
		5021	G	A	A	T		
		5222	T	D	C	D		
	Nsp4	5825	G	R	G	R	D(C/A)	P/Q
	Nsp5	6324	G	E	A	E		
		6357	T	F	C	S	T	F
	Nsp7	7300	G	V	A	I		
	Nsp8	7563	T	L	C	L		
ORF1b	Nsp9	8946	C	H	T	Y		
		9061	C	S	C	S	T	S
		9306	A	T	G	A		
		9363	G	V	C	L		
		9735	C	P	T	S	C	P
	Nsp10	9909	G	V	T	F		
		10,039	G	R	A	K		
		10,207	T	V	C	A		
		10,211	C	P	T	P		
		10,650	A	K	G	E		
	Nsp11	11,354	A	G	A	G		
	Nsp12	12,012	T	Y	C	H		
ORF2	GP2	12,090	G	L	T	F		
		12,333	T	T	C	T	T	T
ORF3	GP3	13,031	C	F	T	F		
		13,397	A	A	T	A		
ORF4	GP4	13,397	A	N	T	Y		
		13,697	A	M	G	V		
		13,762	A	S	T	C		
ORF5	GP5	13,779	T	L	C	L		
		14,020	C	L	T	L		
		14,102	A	S	T	C		
		14,161	G	L	A	L		
		14,180	T	Y	C	H		
		14,317	T	L	A	L		
ORF6	M	14,440	A	T	G	A	A	T
		14,467	G	V	A	I		
		14,662	T	L	C	L		
ORF7	N	15,050	A	T	G	A		
		15,225	T	V	C	A		
		15,227	A	T	G	A		
		15,227	A	T	G	A		

N = nucleotide; AA = amino acid.

NADC-8 (2) virus was more virulent than the NADC-8 (252P) virus and the NADC-8 (251) virus was the least virulent.

Genomic sequencing

We hypothesized the in vivo phenotypic differences observed in the animal study were related to genetic differences among the three passages of the NADC-8 PRRSV strain. To test this hypothesis, the entire genomes were obtained as overlapping PCR products and directly sequenced on both strands with three to six times overlap. For each terminus, 15 independent PCR products were cloned and sequenced. For the sake of this paper, the nomenclature describing PRRSV genomic organization and properties will follow the convention of others (Allende et al., 1999; Nelsen et al., 1999; Snijder and Meulenberg, 1998; Wooten et al., 2000; Yuan et al., 2001). In all three NADC-8 passages, there were two large ORFs, ORF1a and ORF1b, located immediately downstream of the 5′NCR. In the ORF1a–ORF1b frameshift region, the heptanucleotide "slippery" sequence (5′-UUUAAAC-3′) was present in each passage of the NADC-8 virus in the region 7677–7683, four nucleotides upstream of the UAG stop codon of ORF1a. ORFs 2–7 were located downstream of the ORF1b, followed by a 3′NCR and poly(A) tail. No changes were found in 5′and 3′noncoding regions of the three NADC-8 passages.

Nucleotide sequence differences between passages 2 and 251 consisted of 50 nucleotide changes and a three-nucleotide deletion (Table 2). Twenty mutations were silent and thirty mutations resulted in predicted amino acid changes. No nucleotide changes were found in the predicted Nsp4 and Nsp 6 cleavage product of ORF1a. The mutations in ORF1a resulting in predicted amino acid changes were in Nsp1, Nsp2, Nsp3, Nsp5, and Nsp7 cleavage products and only a silent mutation was detected in the Nsp8 cleavage product. There is no mutation in Nsp4 between passages 2 and 251. The three-nucleotide deletion in ORF1a (nucleotide positions 3763–3765) resulted in the loss of a leucine in Nsp2 cleavage product. The nucleotide changes in ORF1b resulted in amino acid changes in Nsp9, Nsp10, and Nsp12 cleavage products. There is no mutation in Nsp11 between passages 2 and 251. There are 17 mutations in the structural genes ORFs 2–7. Both nucleotide changes in ORF3 as well as six additional mutations in ORFs 2 and 4–7 were silent. One or more predicted amino acid changes were predicted for gp2, gp4, gp5, M, and N proteins.

Nucleotide sequence differences between passages 251 and 252p consisted of eight nucleotide changes (Tables 2 and 3). Two of these mutations at positions 9061 (ORF1a) and 12333 (ORF2) were silent. Three of these nucleotide changes at positions 6357 (ORF1a), 9735 (ORF1b), and 14440 (ORF6) led to predicted amino acid changes in Nsp5, Nsp10, and M proteins, respectively. They were direct reversions to genotype NADC8-2. The remaining three nucleotide changes at positions 837, 840, and 5825 (all in ORF1a) were new mutations that produced predicted amino acid changes in Nsp1 and Nsp4 proteins.

Sequencing of the overlapping PCR fragments provided a "consensus" genomic sequence that was not dependent on individual mutations in the minor fractions of the viral genomic pool and PCR products. However, in some instances, nucleotide positions were difficult to deduce and are recorded as mixed results in Table 2, that is, nucleotide positions 1978, 2042, 3166, and 5825. This difficulty may be attributed to a mixture of nucleotides at one position, thus supporting the concept of PRRSV quasi-species.

Discussion

In this study, the full-length nucleotide sequence of the NADC-8 PRRSV strain, passages 2, 251, and 252P, were compared and animal studies demonstrated that the passage 2 virus was the most virulent, passage 251 virus the least virulent, and passage 252P had an intermediate virulence. The first two viruses were passed in cell culture 2 and 251 times each, respectively. Pigs inoculated with the NADC-8 (2) virus developed mild to moderate clinical disease similar to previous studies in which pigs were exposed to NADC-8 (3) virus, virus that had been passed three times in cell culture (Lager et al., 1997, 1999; Mengeling et al., 1996). In addition, the magnitude and duration of virus replication in these pigs was also similar to the previous studies. In contrast, pigs inoculated with the NADC-8 (251) virus did not develop any clinical disease nor was there any

Table 3
Nucleotide and amino acid changes found between passages 251 and 252p

Gene	Nucleotide position within NADC8-2 genome	Protein	NADC8-2		NADC8-251		NADC8-252p	
			Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
ORF1a	837	Nsp1	A	glutamic acid	A	glutamic acid	G	glycine
ORF1a	840	Nsp1	G	glycine	G	glycine	T	valine
ORF1a	5825	Nsp4	G	arginine	G	arginine	D(C/A)	proline/glutamine
ORF1a	6357	Nsp5	T	phenylalanine	C	serine	T	phenylalanine
ORF1a	9061	Nsp9	C	serine	C	serine	T	serine
ORF1b	9735	Nsp10	C	proline	T	serine	C	proline
ORF2	12,333	GP2	T	threonine	C	threonine	T	threonine
ORF6	14,440	M	A	threonine	G	alanine	A	threonine

evidence of virus replication in those pigs, suggesting that none of the pigs became infected following oronasal inoculation with 2×10^4 $CCID_{50}$ virus. The possibility that none of the pigs became infected is supported by previous studies. In one experiment, two of six pigs apparently did not become infected following intramuscular injection of 2×10^6 $CCID_{50}$ NADC-8 (251) PRRSV (Mengeling et al., 2003). This phenomenon was observed in two additional treatment groups in that experiment in which one of six pigs that received NADC-9 (251) and one of six pigs that received NVSL-14 (251) apparently did not become infected following a similar intramuscular injection of highly attenuated PRRSV. Although four, five, and five pigs did become infected with NADC-8 (251), NADC-9 (251), and NVSL-14 (251) PRRSV, respectively, none of them developed clinical signs of disease and all of them seroconverted. In a separate series of animal studies, some fetuses (50–90 days of gestational age) were apparently not infected with the NADC-8 (251) virus following direct intramuscular injection or intraamniotic injection of the fetus with about 1×10^3 $CCID_{50}$ virus (Lager and Butler, 2002; unpublished observations). However, some fetuses were infected and they appeared normal despite replication of the virus.

The NADC-8 (252P) PRRSV used in this study was derived from a pig that was subclinically infected with the NADC-8 (251) PRRSV (unpublished observations). The pig was inoculated with approximately 2×10^6 $CCID_{50}$ NADC-8 (251) PRRSV at about 4 weeks of age. PRRSV was isolated from blood collected 28 days after inoculation. Because this virus had been replicating in the pig for 28 days, we hypothesized that when compared to the NADC-8 (251) passage, this virus may have an increased propensity to replicate in pigs or an increase in virulence. This supposition was confirmed in the animal experiment reported here.

In the previously described animals studies, the same source herd was used for the pigs and pregnant sows. All of the pigs were of a similar age, health status, and genetics (Yorkshire × Chester White cross) although they were purchased at different times. Perhaps this temporal effect might explain the apparent lack of infectivity found in the present study for the NADC-8 (251) PRRSV. However, a dose effect or route of administration may be a more probable explanation as to why the 16 pigs oronasally exposed to 2×10^4 $CCID_{50}$ virus apparently did not become infected although most pigs intramuscularly exposed to 2×10^6 $CCID_{50}$ virus did become infected.

Terminal parts of the viral genome are usually crucial for replication and transcription of viral RNA and are highly conserved 5′and 3′regions (Boyer and Haenni, 1994; Klump et al., 1990; Sarnow, 1989). This was the case for the three passages of the NADC-8 strain. No nucleotide changes were found in the 5′ and 3′ ends of the three NADC-8 passages, even after 251 passages in cell culture and replication in a pig for 4 weeks. Based on previous reports comparing an attenuated PRRSV with its wild-type parental virus (Allende et al., 2000; Yuan et al., 2001), genetic differences were anticipated between passages 2 and 251 viruses. In this PRRSV attenuation model using the NADC-8 strain, 50 nucleotide mutations and a three-nucleotide deletion were found between passages 2 and 251 viruses (Table 2). This mutation rate is somewhat similar to the mutation rate reported for the wild-type PRRSV that was attenuated by repetitive cell culture passage and used as a commercially available vaccine. In that case, when the attenuated virus was compared to the parental virus there were 44 nucleotide changes detected after 70 in vitro passages (Yuan et al., 2001). When the location of mutations in these two examples of attenuation of wild-type PRRSV by cell culture adaptation are examined, there is no obvious site in the genome associated with attenuation. As might be expected for a random incidence of mutation, there would be more mutations in larger ORFs when compared to smaller ORFs. This proportionate incidence of mutation was found in this study with the NADC-8 strain and with the attenuation of VR-2332 into a vaccine strain (Yuan et al., 2001) (Table 4). Although the mutations appear random, it is assumed that one or more of them relate to the attenuation process.

In an attempt to locate a mutation site that may relate to the attenuation process, the least virulent virus, the 251st passage, was propagated in a pig with the anticipation that any mutations occurring during the pig passage might be related to a loss of attenuation, that is, an increase in virulence. When comparing the nucleotide sequences of passages 251 and 252P, there were 8 nucleotide differences (Tables 2 and 3). Six of these led to amino acid changes, 3 of which were revertants to the wild-type genotype. The two nucleotide changes that did not result in an amino acid change were in ORF1a and ORF2. Five mutations resulting in amino acid changes were in ORF1a and ORF1b. In ORF1a mutations at positions 837, 840, 5825, and 6357, resulted in deduced amino acid changes in Nsp1, 1, 4, and 5, respectively. Nsp1 has two papain-like protein domains

Table 4
Number of mutations acquired by the PRRSV strains NADC-8 and VR-2332 following attenuation via cell culture passage

Genomic region	Number of nucleotide changes		Percent homology between parent and attenuated virus	
	NADC-8	VR-2332	NADC-8	VR-2332
5′-UTR	0[a]	2[b]	100[a]	99.5[b]
ORF1a	22	15	99.7	99.8
ORF1b	11	13	99.7	99.7
ORF2	1	4	99.9	99.5
ORF3	2	4	99.7	99.1
ORF4	3	2	99.7	99.6
ORF5	6	2	99.0	99.7
ORF6	2	3	99.6	99.4
ORF7	3	0	99.2	100
3′-UTR	0	0	100	100

[a] Data from the NADC-8 PRRSV strain of PRRSV.
[b] Data from the VR-2332 PRRSV strain (Yuan et al., 2001).

(α and β). The mutation in 837 and 840 nucleotide positions resulted in amino acid changes in Nsp1β domains containing a putative papain-like cysteine protease. The mutation in 5825 nucleotide position is located between the sequence of predicted chymotrypsin-like serine protease cleavage sites in Nsp4. The mutation in 6357 nucleotide position is located close to the chymotrypsin-like serine protease cleavage sites of Nsp5 protein. In ORF1b mutation in position 9735 resulted in a deduced amino acid change in Nsp10, a protein containing the zinc-finger and helicase domains. The remaining mutation was at position 14440 in ORF 6 resulting in an amino acid change in the predicted membrane-spanning region (amino acid 18-34) of the membrane or M protein. The predicted amino acid changes in Nsp5, Nsp10 and M proteins were all revertants to the original wild-type virus sequence, the NADC-8 (2) virus.

Although there are many reports describing complete and partial genomic sequences of PRRSV strains, there are only a few that analyze the mutations associated with attenuation of PRRSV and the reversion of attenuated PRRSV to a more virulent state. The incidence and clustering of mutations in ORF1 associated with reversion to virulence found in this study have been observed before when vaccine-derived field PRRSV isolates were sequenced and compared with the vaccine parental strain, VR-2332 (Nielsen et al., 2001). Two reversions to the VR-2332 genotype were consistently found in these field isolates: one was in the papain-like cysteine protease domain of Nsp1b, and the other one in the helicase domain of the Nsp10 protein. In addition, the reversion in ORF 6 found in this study supports observations by others who associated mutations in ORF 6 with a reversion to virulence (Madsen et al., 1998). A full-length genetic analysis of a virulent PRRSV field isolate (16244B) revealed that at the nucleotide level, it has about 98% homology with the VR-2332 PRRSV strain and the attenuated vaccine derived from VR-2332 (Allende et al., 2000). Based on this work, it is proposed that the 16244B virus is a descendent of the vaccine and genetic differences between the 16244B and the vaccine virus could reflect mutations in vaccine virus that led to a reversion to virulence. Mutations were identified in ORFs 1a (Nsp1b, Nsp2) and 1b (Nsp1, 2, 3, 5, and 6) that may have contributed to a reversion to virulence. However, the exact origin of the 16244B virus is not clear which may cast doubt on the linkage between these specific mutations and a reversion to virulence (Yuan et al., 2001).

Collectively, this study and the work of others suggest that mutations in Nsp1, Nsp10, and M protein may be related to the attenuation of PRRSV by repetitive passage in cell culture. Confirmation of this assumption awaits the necessary experiments utilizing a reverse genetics system for PRRSV. Additional mutations between passages 251and 252p viruses were found in this study and, as in other studies, the influence of these mutations on PRRSV virulence is not known.

Materials and methods

Virus

The NADC-8 strain of PRRSV used in this study was isolated from the serum of a weak-born congenitally infected pig (Lager et al., 1997). Selection of each virus passage used for experimental inoculation of pigs was based on the relative reported or presumed virulence, mainly the degree of replication in pigs and cell cultures. Passage level 2 had been passaged once in cell culture following its initial isolation in cell culture. Although limited animal studies have been conducted with the second passage of NADC-8 PRRSV, it is assumed to be virulent based on extensive studies with the third passage of NADC-8 PRRSV (Lager et al., 1997, 1999; Mengeling et al., 1996). Passage level 251 was derived from passage level 2 by an additional 249 passages in cell culture. Passage level 251 was highly attenuated producing limited if any clinical effects in infected pigs (Mengeling et al., 2003). Sometimes pigs did not become infected even when passage 251 was administered intramuscularly at a dose of 2×10^6 median cell culture infectious doses ($CCID_{50}$) (Mengeling et al., 2003). Passages 3–13 were made at 3- or 4-day intervals in either 75 cm^2 tissue culture flasks or 24-well tissue culture plates. Between passages 14 and 249, the virus was passaged in 24-well plates at daily intervals at or near the maximum dilution that resulted in infection of the next culture in the passage series. The maximum dilution was identified in retrospect from among several dilutions made at each passage. Because the efficiency of virus replication in cell culture increased with passage level, the passage dilution (i.e., the dilution of medium from the previous culture) ranged from 1:5 for early passages to 1:50,000 for late passages. The indicated dilution was based on the final dilution of inoculum in the medium of the culture to which the inoculum was added. Passage level 252P was derived from passage level 251 by infecting a pig with an aliquot of the 251st cell culture passage, collecting a virus-containing serum sample 28 days later (presumably enough time to allow 84 cycles of virus replication in the pig), and then passing that sample (virus population) once more in cell culture. We presumed that passage level 252P might be more virulent than passage level 251 because the 252P virus had replicated in a pig for 4 weeks and this virus might have accumulated mutations that would promote viral replication in vivo.

Viral inocula were prepared by freezing and thawing the virus-infected cell culture when approximately 50% cytopathic effect was visible. The cell lysate was clarified, titrated, and the inoculum was diluted to provide a virus solution containing 1×10^4 $CCID_{50}$/ml. A sham or mock-infected cell culture lysate was prepared in a similar fashion.

Animal study

An animal study was conducted to compare the virulence of the three passages of the NADC-8 PRRSV strain under

similar conditions. Sixty-four conventionally raised 4-week-old crossbred piglets (Yorkshire × Chester White) were randomly divided into four treatment groups, 16 pigs to a group. Each treatment group was housed individually in animal isolation facilities at the National Animal Disease Center. On Day 0, Groups 1, 2, 3, and 4 received a 2-ml oronasal inoculation of the sham, (251), (252P), and (2) passages of the NADC-8 PRRSV strain, respectively. Four pigs out of each treatment group were euthanized on experiment days 3, 10, 17, and 35 with an intravenous injection of pentobarbital. Blood was collected from all pigs on days 0, 3, 10, 17, and 35. For the purposes of this study, the virulence of each virus passage was based on the magnitude and duration of virus replication and seroconversion in the inoculated pigs. Each serum sample was tested by virus isolation (Lager et al., 1997) and for the presence of PRRSV antibodies using a commercially available ELISA (IDEXX, USA). Virus titer was determined for virus-positive serum samples and the mean values for virus titer and ELISA tests were compared using ANOVA. A P value ≤ 0.05 was considered significant. The pigs were observed daily for overt signs of illness, that is, anorexia, listlessness, and dyspnea.

Cells, RNA isolation, and amplification of PRRSV genome

The following methods were used for all three passages of the NADC-8 PRRSV strain that were used as inoculum and sequenced. The MARC-145 cell culture was used for isolation and propagation of the virus (Kim et al., 1993). The cells were cultured in Earl's minimum essential medium, supplemented with 10% fetal bovine serum and 50 mg/ml gentamicin sulfate in a humidified 5% CO_2 atmosphere at 37 °C. Total RNA was isolated from monolayers of virus-infected cells with TRIzol reagent (Life Technologies) according to the manufacturer's specification.

Reverse transcription and polymerase chain reaction (RT-PCR) were carried out with reverse transcriptase and TaqI polymerase (Life Technologies) as previously described (Sellner et al., 1994) using 151 specific primers. Primers were designed based on published sequences of PRRSV genomes found under the following Genbank accession numbers: AF046869 (PRRSV strain 16244B), AF030244, and U00153 (PRRSV strain VR-2332). The GC content of the primers was 45–53%, they were 17 and 18 nucleotides long, and intervals between primers on the opposite strands were approximately 100–150 nucleotides. All RT-PCR primer sequences are available from the authors on request. Amplifications were carried out in 100 μl volume containing 1 μg of RNA, 100 pmol of each primer, 5 U of TaqI polymerase, 2.5 U MMLV-RT, 10 mM Tris–HCl, (pH 9.0 at 25 °C), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$. Reverse transcription was carried out for 60 min at 50–53 °C followed by a 5-min holding period at 94 °C. Amplification was carried out for 30 cycles (30 s at 94 °C, 30 s at 51–53 °C, 2 min at 72 °C). PCR products of the 3′ and 5′ ends of the NADC8 genome were obtained using a 3′ RACE and 5′RACE Systems for Rapid Amplification of cDNA ends (Life Technologies).

Sequence analysis

Overlapping PCR products for each virus covering the entire genome were gel-purified using Geneclean PCR purification kits (Geneclean) and directly sequenced using PRIZM FS reagents (Perkin Elmer). The sequencing reactions were run and analyzed in an automated sequencer (ABI 377, USA). Sequences were aligned and analyzed using MacVector/AssemblyLign (Oxford Molecular Group, USA).

References

Allende, R., Lewis, T.L., Lu, Z., Rock, D.L., Kutish, G.F., Ali, A., Doster, A.R., Osorio, F.A., 1999. North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. J. Gen. Virol. 80, 307–315.

Allende, R., Kutish, G.F., Laegried, W., Lu, Z., Lewis, T.L., Rock, D.L., Friesen, J., Galeota, J.A., Doster, A.R., Osorio, F.A., 2000. Mutation in the genome of porcine reproductive and respiratory syndrome virus responsible for the attenuation phenotype. Arch. Virol. 145, 1149–1161.

Botner, A., Strandbygaard, B., Sorensen, K.J., Have, P., Madsen, K.G., Madsen, E.S., Alexandersen, S., 1997. Appearance of acute PRRS-like symptoms in sow herds after vaccination with a modified live PRRS vaccine. Vet. Rec. 141, 497–499.

Boyer, J.C., Haenni, A.L., 1994. Infectious transcripts and cDNA clones of RNA viruses. Virology 198, 415–426.

Cavanagh, D., 1997. *Nidovirales*: a new order comprising *Coronaviridae* and *Arteriviridae*. Arch. Virol. 142, 629–633.

Forsberg, R., Storgaard, T., Nielsen, H.S., Oleksiewicz, M.B., Cordioli, P., Sala, G., Hein, J., Botner, A., 2002. The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. Virology 299, 38–47.

Indik, S., Valicek, L., Klein, D., Klanova, J., 2000. Variations in the major envelope glycoprotein GP5 of Czech strains of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 81, 2497–2502.

Kim, H.S., Kwang, J., Yoon, I.J., Joo, H.S., Frey, M.L., 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogenous subpopulation of MA-104 cell line. Arch. Virol. 133, 477–483.

Klump, W.M., Bergmann, I., Muller, B.C., Ameis, D., Kandolf, R., 1990. Complete nucleotide sequence of infectious coxsachie-virus B3 cDNA: two initial 5′ uridine residues are regained during plus-strand RNA synthesis. J. Virol. 64, 1573–1583.

Lager, K.M., Butler, J.E., 2002. Midgestation fetal PRRSV infection did not induce an immunotolerant state. Proc. 17th Int. Pig Vet. Society, vol. 1, p. 273. Ames, Iowa, USA.

Lager, K.M., Mengeling, W.L., Brockmeier, S.L., 1997. Homologous challenge of porcine reproductive and respiratory syndrome virus immunity in pregnant swine. Vet. Microbiol. 58, 113–125.

Lager, K.M., Mengeling, W.L., Brockmeier, S.L., 1999. Evaluation of protective immunity in gilts inoculated with the NADC-8 isolate of porcine reproductive and respiratory syndrome virus (PRRSV) and challenge-exposed with an antigenically distinct PRRSV isolate. Am. J. Vet. Res. 60, 1022–1027.

Le Gall, A., Legeay, O., Bourhy, H., Arnauld, C., Albina, E., Jestin, A.,

1998. Molecular variation in the nucleoprotein gene (ORF 7) of the porcine reproductive and respiratory syndrome virus (PRRSV). Virus Res. 54, 9–21.

Madsen, K.G., Hansen, C.M., Madsen, E.S., Strandbygaard, B., Botner, A., Sorensen, K.J., 1998. Sequence analysis of porcine reproductive and respiratory syndrome virus of the American type collected from Danish swine herds. Arch. Virol. 143, 1683–1700.

Mardassi, H., Mounir, S., Dea, S., 1995. Molecular analysis of the ORFs 3 to 7 of porcine reproductive and respiratory syndrome virus. Quebec reference strain. Arch. Virol. 140, 1405–1418.

Meng, X.L., Paul, P.S., Halbur, P.C., Lum, M.A., 1995. Phylogenetic analyses of the putative M (ORF6) and N (ORF7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the USA and Europe. Arch. Virol. 140, 745–755.

Mengeling, W.L., Vorwald, A.C., Lager, K.M., Brockmeier, S.L., 1996. Comparison among strains of porcine reproductive and respiratory syndrome virus for their ability to cause reproductive failure. Am. J. Vet. Res. 57, 834–839.

Mengeling, W.L., Lager, K.M., Vorwald, A.C., Koehler, K.J., 2003. Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. Vet. Microbiol. 93, 13–24.

Meulenberg, J.J.M., Hulst, M.M., de Meijer, E.J., Moonen, P.L.J.M., den Besten, A., de Kluyver, E.P., Wensvoort, G., Moormann, R.J.M., 1993. Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology 192, 62–72.

Murtaugh, M.P., Elam, M.R., Kakach, L.T., 1995. Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. Arch. Virol. 140, 1451–1460.

Nelsen, C.J., Murtaugh, M.P., Faaberg, K.S., 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J. Virol. 73, 270–280.

Nielsen, H.S., Oleksiewicz, M.B., Forsberg, R., Stadejek, T., Botner, A., Storgaard, T., 2001. Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. J. Gen. Virol. 82, 1263–1272.

Nielsen, J., Botner, A., Bille-Hansen, V., Oleksiewicz, M.B., Storgaard, T., 2002. Experimental inoculation of late term pregnant sows with a field isolate of porcine reproductive and respiratory syndrome vaccine-derived virus. Vet. Microbiol. 84, 1–13.

Sarnow, P., 1989. Role of 3′-ends sequences in infectivity of poliovirus transcripts made in vitro. J. Virol. 63, 467–470.

Sellner, L.N., Coelen, R.J., Mackenzie, J.S., 1994. Sensitive detection of Ross River virus a one-tube nested RT-PCR. J. Virol. Methods 49, 47–58.

Shen, S., Kwang, J., Liu, W., Liu, D.X., 2000. Determination of the complete nucleotide sequence of a vaccine strain of porcine reproductive and respiratory syndrome virus and identification of the Nsp2 gene with a unique insertion. Arch. Virol. 145, 871–873.

Snijder, E.J., Meulenberg, J.J.M., 1998. The molecular biology of arteriviruses. J. Gen. Virol. 79, 961–979.

Stadejek, T., Stankevicius, A., Storgaard, T., Oleksiewicz, M.B., Belak, S., Drew, T.W., Pejsak, Z., 2002. Identification of radically different variants of porcine reproductive and respiratory syndrome virus in Eastern Europe: towards a common ancestor for European and American viruses. J. Gen. Virol. 83, 1861–1873.

Storgaard, T., Oleksiewicz, M., Botner, A., 1999. Examination of the selective pressures on a live PRRS vaccine virus. Arch. Virol. 144, 2389–2401.

Suarez, P., Zardoya, R., Martin, M.J., Prieto, C., Dopazo, J., Solana, A., Castro, J.M., 1996. Phylogenetic relationship of European strain of porcine reproductive and respiratory syndrome virus (PRRSV) inferred from DNA sequences of putative ORF-5 and ORF-7 genes. Virus Res. 42, 159–165.

Wensvoort, G., Terpstra, C., Pol, J., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., Broekhuijsen, J.M., Moonen, P.L.J.M., Zetstra, T., de Boer, E.A., Tibben, H.J., de Jong, M.F., van't Veld, P., Groenland, G.J.R., van Gennep, J.A., Voets, M.T.h., Verheijden, J.H.M., Braamskamp, J., 1991. Mystery swine disease in the Netherlands: the isolation of Lelystad virus. Vet. Q. 13, 121–130.

Wooten, S., Yoo, D., Rogan, D., 2000. Full-length sequence of a Canadian porcine reproductive and respiratory syndrome virus (PRRSV) isolate. Arch. Virol. 145, 2297–2323.

Yuan, S., Mickelson, D., Murtaugh, M.P., Faaberg, K.S., 2001. Complete genome comparison of porcine reproductive and respiratory syndrome virus parental and attenuated strains. Virus Res. 74, 99–110.